FORM PTO-1390 (REV 11-2000)	U.S. DEPARTMENT OF COMMERCE PATENT AND TRADEMARK OFFICE	ATTORNEY'S DOCKET NUMBER 3557-11
TRANSMITTAL LETTER TO THE UNITED STATES DESIGNATED/ELECTED OFFICE (DO/EO/US) CONCERNING A FILING UNDER 35 U.S.C. 371		U.S. APPLICATION NO. (If known, see 37 C.F.R. 1.5) 10/088027 Unknown
INTERNATIONAL APPLICATION NO. **PCT/EP00/09245**	INTERNATIONAL FILING DATE **21 September 2000**	PRIORITY DATE CLAIMED **1 October 1999**

TITLE OF INVENTION

GMP SYNTHETASE DERIVED FROM PLANTS

APPLICANT(S) FOR DO/EO/US

LERCHL et al

Applicant herewith submits to the United States Designated/Elected Office (DO/EO/US) the following items and other information:

1. ☒ This is a **FIRST** submission of items concerning a filing under 35 U.S.C. 371.
2. ☐ This is a **SECOND** or **SUBSEQUENT** submission of items concerning a filing under 35 U.S.C. 371.
3. ☒ This is an express request to begin national examination procedures (35 U.S.C. 371(f)). The submission must include items (5), (6), (9) and (21) indicated below.
4. ☒ The U.S. has been elected by the expiration of 19 months from the priority date (Article 31).
5. A copy of the International Application as filed (35 U.S.C. 371(c)(2)).
   - a. ☐ is attached hereto (required only if not communicated by the International Bureau).
   - b. ☒ has been communicated by the International Bureau.
   - c. ☐ is not required, as the application was filed in the United States Receiving Office (RO/US).
6. ☒ An English language translation of the International Application as filed (35 U.S.C. 371(c)(2)).
   - a. ☒ is attached hereto.
   - b. ☐ has been previously submitted under 35 U.S.C. 154(d)(4).
7. ☐ Amendments to the claims of the International Application under PCT Article 19 (35 U.S.C. 371(c)(3))
   - a. ☐ are attached hereto (required only if not communicated by the International Bureau).
   - b. ☐ have been communicated by the International Bureau.
   - c. ☐ have not been made; however, the time limit for making such amendments has **NOT** expired.
   - d. ☐ have not been made and will not be made.
8. ☐ An English language translation of the amendments to the claims under PCT Article 19 (35 U.S.C. 371(c)(3)).
9. ☒ An oath or declaration of the inventor(s) (35 U.S.C. 371(c)(4)).
10. ☒ A English language translation of the annexes of the International Preliminary Examination Report under PCT Article 36 (35 U.S.C. 371(c)(5)).

Items 11 To 20 below concern document(s) or information included:

11. ☐ An Information Disclosure Statement under 37 C.F.R. 1.97 and 1.98.
12. ☒ An assignment document for recording. A separate cover sheet in compliance with 37 C.F.R. 3.28 and 3.31 is included.
13. ☒ A FIRST preliminary amendment.
14. ☐ A SECOND or SUBSEQUENT preliminary amendment.
15. ☐ A substitute specification.
16. ☐ A change of power of attorney and/or address letter.
17. ☒ A computer-readable form of the sequence listing in accordance with PCT Rule 13ter.2 and 35 U.S.C. 1.821-1.825.
18. ☐ A second copy of the published international application under 35 U.S.C. 154(d)(4).
19. ☐ A second copy of the English language translation of the international application under 35 U.S.C. 154(d)(4).
20. ☒ Other items or information. PTO-1449 and copy of International Search Report and Statement with paper and computer readable copies of Sequence Listing

U.S. APPLICATION NO. (If known, see 37 C.F.R. 1.5)	INTERNATIONAL APPLICATION NO.	ATTORNEY'S DOCKET NUMBER
10/088027 Unknown	PCT/EP00/09245	3557-11

21. ☒ The following fees are submitted:

	CALCULATIONS PTO USE ONLY		
BASIC NATIONAL FEE (37 C.F.R. 1.492(a)(1)-(5):			
-- Neither international preliminary examination fee (37 C.F.R. 1.482) nor international search fee (37 C.F.R. 1.445(a)(2)) paid to USPTO and International Search Report not prepared by the EPO or JPO $1040.00			
-- International preliminary examination fee (37 C.F.R. 1.482) not paid to USPTO but International Search Report prepared by the EPO or JPO $890.00			
-- International preliminary examination fee (37 C.F.R. 1.482) not paid to USPTO but international search fee (37 C.F.R. 1.445(a)(2)) paid to USPTO $740.00			
-- International preliminary examination fee (37 C.F.R. 1.482) paid to USPTO but all claims did not satisfy provisions of PCT Article 33(1)-(4) $710.00			
-- International preliminary examination fee (37 C.F.R. 1.482) paid to USPTO and all claims satisfied provisions of PCT Article 33(1)-(4) $100.00			
ENTER APPROPRIATE BASIC FEE AMOUNT =	$	890.00	
Surcharge of $130.00 for furnishing the oath or declaration later than ☐ 20 ☐ 30 months from the earliest claimed priority date (37 C.F.R. 1.492(e)).	$	0.00	

CLAIMS	NUMBER FILED		NUMBER EXTRA	RATE			
Total Claims	25	-20 =	5	X	$18.00	$	90.00
Independent Claims	5	-3 =	2	X	$84.00		168.00
MULTIPLE DEPENDENT CLAIMS(S) (if applicable)					$280.00	$	280.00
					TOTAL OF ABOVE CALCULATIONS =	$	**1428.00**
☐ Applicant claims small entity status. See 37 CFR 1.27. The fees indicated above are reduced by 1/2.							0.00
					SUBTOTAL =	$	**1428.00**
Processing fee of $130.00, for furnishing the English Translation later than ☐ 20 ☐ 30 months from the earliest claimed priority date (37 C.F.R. 1.492(f)).					+		0.00
					TOTAL NATIONAL FEE =	$	**1428.00**
Fee for recording the enclosed assignment (37 C.F.R. 1.21(h)). The assignment must be accompanied by an appropriate cover sheet (37 C.F.R. 3.28, 3.31). **$40.00** per property					+	$	40.00
Fee for Petition to Revive Unintentionally Abandoned Application ($1280.00 – Small Entity = $640.00)						$	0.00
					TOTAL FEES ENCLOSED =	$	**1468.00**
						Amount to be: refunded	$
						Charged	$

a. ☒ A check in the amount of $1468.00 to cover the above fees is enclosed.
b. ☐ Please charge my Deposit Account No. 14-1140 in the amount of $_____ to cover the above fees. A duplicate copy of this form is enclosed.
c. ☒ The Commissioner is hereby authorized to charge any additional fees which may be required, or credit any overpayment to Deposit Account No. 14-1140. A duplicate copy of this form is enclosed.
d. ☒ The entire content of the foreign application(s), referred to in this application is/are hereby incorporated by reference in this application.

NOTE: Where an appropriate time limit under 37 C.F.R. 1.494 or 1.495 has not been met, a petition to revive (37 C.F.R. 1.137(a) or (b)) must be filed and granted to restore the application to pending status.

SEND ALL CORRESPONDENCE TO:

NIXON & VANDERHYE P.C.
1100 North Glebe Road, 8th Floor
Arlington, Virginia 22201-4714
Telephone: (703) 816-4000

SIGNATURE

B. J. Sadoff
NAME

36,663 REGISTRATION NUMBER

March 14, 2002 Date

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

In re Patent Application of

LERCHL et al Atty. Ref.: **3557-11**

Serial No. **Unknown** Group:

National Phase of: **PCT/EP00/09245**
International Filing Date: **21 September 2000**

Filed: **March 14, 2002** Examiner:

For: **GMP SYNTHETASE DERIVED FROM PLANTS**

* * * * * * * * * * * *

March 14, 2002

Assistant Commissioner for Patents
Washington, DC 20231

Sir:

PRELIMINARY AMENDMENT

Prior to calculation of the filing fee and in order to place the above identified application in better condition for examination, please amend as follows:

IN THE SPECIFICATION

Page 1, after the title insert the following:

-- This application is the US national phase of international application PCT/EP00/09245 filed September 21, 2000 which designated the U.S. --.

Insert the attached Sequence Listing in place of the originally-filed Sequence Listing.

IN THE CLAIMS

Please substitute the following amended claims for corresponding claims previously presented. A copy of the amended claims showing current revisions is attached.

14. (Amended) An inhibitor of plant GMP synthetase identified using an assay system as claimed in claim 11.

15. (Amended) An inhibitor as claimed in claim 13 for use as herbicide.

Please add the following new claims:

17. (New) An inhibitor of plant GMP synthetase identified using an assay system as claimed in claim 12.

18. (New) An inhibitor as claimed in claim 14 for use as herbicide.

REMARKS

Attached hereto is a marked-up version of the changes made to the claims by the current amendment. The attached page is captioned "**Version with markings to show changes made**."

The attached paper and computer readable copies of the Sequence Listing are the same. No new matter has been added. A separate Statement to this effect is attached.

The above amendments are made to place the claims in a more traditional format.

Respectfully submitted,

NIXON & VANDERHYE P.C.

By: ____________________

B. J. Sadoff
Reg. No. **36,663**

BJS:lmy

1100 North Glebe Road, 8th Floor
Arlington, VA 22201-4714
Telephone: (703) 816-4000
Facsimile: (703) 816-4100

VERSION WITH MARKINGS TO SHOW CHANGES MADE

14. (Amended) An inhibitor of plant GMP synthetase identified using an assay system as claimed in claim 11 [or 12].

15. (Amended) An inhibitor as claimed in [either of claims 13 or 14] <u>claim 13</u> for use as herbicide.

4/prts

GMP synthetase from plants

The present invention relates to the identification of plant GMP synthetase (guanosine-monophosphate synthetase) as novel target for herbicidal agents. The present invention further relates to DNA sequences coding for a polypeptide having GMP synthetase (EC 6.3.5.2) activity. The invention moreover relates to the use of a nucleic acid coding for a protein having GMP synthetase activity of plant origin for producing an assay system for identifying inhibitors of GMP synthetase having a herbicidal action, and to inhibitors of plant GMP synthetase identified using this assay system. The invention further relates to the use of the nucleic acid coding for plant GMP synthetase for producing plants with increased resistance to inhibitors of GMP synthetase, and for producing plants with a modified content of guanosine nucleotides. The invention additionally relates to a method for eliminating unwanted plant growth, which comprises treating the plants to be eliminated with a compound which specifically binds to GMP synthetase encoded by a DNA sequence SEQ-ID No. 1 or a DNA sequence hybridizing with the latter, and inhibits the function thereof.

Plants are able to synthesize their cellular components from carbon dioxide, water and inorganic salts.

This process is possible only through the use of biochemical reactions to synthesize organic substances. It is necessary for plants to synthesize de novo the nucleotides as constituents of nucleic acids.

Especially in rapidly growing plant tissues it is necessary for nucleotides as constituents of the nucleic acids DNA and RNA to be synthesized by multistage metabolic pathways. Nucleotides are moreover linked in with virtually all metabolic pathways. Nucleoside triphosphates, especially ATP, drive many energy-expending reactions in cells. Adenine nucleotide additionally occurs as component in essential coenzymes such as coenzyme A and nicotinamide and flavin coenzymes, which are involved in many cellular conversions. Guanosine nucleotides give a reaction direction to various cellular processes such as protein translation, microtubule assembly, vesicular transport, signal transduction and cell division. In addition, nucleotides are the starting metabolites for the biosynthesis of methylxanthines such as caffeine and theobromine, especially in the Rubiaceae and Theaceae families of plants.

Purine nucleotides are formed in microorganisms, animals and plants de novo in the same way starting from phosphoribosyl pyrophosphate (PRPP). IMP is synthesized in a 10-stage reaction sequence. IMP can be converted in subsequent reactions by adenylosuccinate synthetase and adenylosuccinate lyase into AMP. In the synthesis of GMP there is initial conversion of IMP by IMP dehydrogenase into XMP which is aminated by GMP synthetase to give GMP, see Fig. 1.

Genes which code for GMP sythetase [sic] have been isolated from various organisms.

The compartmentation of the purine biosynthetic pathway in plants has not to date been extensively investigated. The nitrogen which is fixed in the form of glutamine and aspartate in the root nodules of legumes is firstly converted via the de novo synthetic pathway into purines. This pathway is localized in the plastids in the root nodules of *Glycine max* and *Vigna unguiculata* L. (Boland and Schubert, Arch. Biochem. Biophys. 220 (1983), 179-187; Shelp et al., Arch. Biochem. Biophys. 224 (1983), 429-441). However, more recent investigations have shown that enzyme activitites of the purine biosynthetic pathway are also to be found in mitochondria in the root nodules of *Vigna ungiculata* [sic] (Atkins et al., Plant Physiology 113 (1997), 127-135; Smith et al., Plant Molecular Biology 36 (1998), 811-820).

The regulation of this synthetic pathway has to date been investigated only in microorganisms and animals and comprises transcription control, end-product inhibition and allosteric regulation. The enzyme PRPP amidotransferase (PRPP ATAse) of the second reaction step is attributed with a key position in the animal as well as the plant system and is subject to allosteric regulation by the end products IMP, AMP and GMP (Reynolds et al., Archives of Biochemistry and Biophysics 229 (1984), 623-631).

GMP-Synthetase also plays a part in relation to the balanced synthesis of guanosine nucleotides and adenosine nucleotides because ATP is a substrate of GMP synthetase.

Since plants are dependent on a functioning nucleotide metabolism, this metabolism is obviously a possible target of novel herbicides. In fact, agents with an inhibitory effect on enzymes of de novo purine biosynthesis have already been described. An example which may be mentioned is 5'-phosphohydantocidin which inhibits an enzyme of plant purine metabolism, adenylosuccinate synthetase (ASS) (Siehl et al., Plant Physiol. 110 (1996), 753-758). Inhibitors for enzymes of

this metabolic pathway also exist from animals and microorganisms. Folate analogs inhibit various folate-dependent reactions, inter alia the enzyme GAR transformylase and have antiproliferative, antiinflammatory and immunosuppressant effects. Mycophenolate (MPA), as an inhibitor of IMP dehydrogenase, has antimicrobial, antiviral and immunosuppressant effects. (Kitchin et al., Journal of the American Academy of Dermatology 37 (1997), 445-449).

Demonstration of the suitability of an enzyme as herbicide target can be shown, for example, by reducing the enzyme activity by means of the antisense technique in transgenic plants. If reduced growth is brought about in this way, it can be concluded that the enzyme whose activity has been reduced is a suitable site of action of herbicidal agents. This has been shown by way of example for acetolactate synthase on transgenic potato plants (Höfgen et al., Plant Physiology 107 (1995), 469-477).

It is an object of the present invention to prove that GMP synthetase in plants is a suitable herbicidal target, to isolate a complete plant cDNA coding for the enzyme GMP synthetase and functional expression thereof in bacterial or eukaryotic cells, and to produce an efficient and simple GMP synthetase assay system for carrying out the inhibitor-enzyme binding studies.

We have found that this object is achieved by isolation of a gene coding for the plant enzyme GMP synthetase, the production of antisense constructs of GMP synthetase, and functional expression of the GMP synthetase in bacterial or eukaryotic cells.

One aspect of the present invention relates to the isolation of a full-length cDNA coding for a functional glutamine-hydrolyzing GMP synthetase (EC 6.3.5.2.) from tobacco (*Nicotiana tabacum*).

A first aspect of the present invention is a DNA sequence SEQ-ID NO:1 comprising the coding region of a plant GMP synthetase from tobacco, see Example 1.

Another aspect of the invention is a DNA sequence SEQ-ID No. 3 comprising a portion of the coding region of a plant GMP synthetase from Physcomitrella patens, see Example 2.

Further aspects of the invention are DNA sequences which are derived from SEQ-ID NO: 1 or SEQ-ID No: 3 or hybridize with one of these sequences and code for a protein which has the biological activity of a GMP synthetase.

Tobacco plants of the line Nicotiana tabacum cv. Samsun NN harboring an antisense construct of GMP synthetase have been characterized in detail. The plants show growth retardation to differing extents. The transgenic lines, and the progeny as 1st and 2nd generation showed reduced growth in soil. In plants with reduced growth it was possible to detect a reduced, compared with the wild-type, GMP-7M RNA amount in the Northern hybridization. It was also possible in a Western blot experiment to detect a reduced amount, compared with wild-type plants, of GMP synthetase in the transgenic lines, see Example 7. A correlation can be found in the growth retardation and reduction in the amount of GMP synthetase protein. This clear association demonstrates for the first time that GMP is unambiguously a suitable target protein for herbicidal agents.

In order to be able to find efficient inhibitors of plant GMP synthetase, it is necessary to provide suitable assay systems wih which inhibitor-enzyme binding studies can be carried out. For this purpose, for example, the complete cDNA sequence of the GMP synthetase from tobacco is cloned in an expression vector (pQE, Qiagen) and is expressed in E. coli, see Example 4.

An alternative possibility is, however, to express the expression cassette comprising a DNA sequence SEQ-ID No. 1 for example in other bacteria, in yeasts, fungi, algae, plant cells, insect cells or mammalian cells, see Example 5.

The GMP synthetase protein expressed with the aid of the expression cassette according to the invention is particularly suitable for finding inhibitors specific for GMP synthetase.

For this purpose, the plant GMP synthetase can be employed, for example, in an enzyme assay in which the activity of the GMP synthetase is measured in the presence and absence of the agent to be tested. Qualitative and quantitative information about the inhibitory characteristics of the agent to be tested is obtainable from comparison of the two activity determinations, see Example 8.

The assay system according to the invention can be used for rapid and simple testing of a large number of chemical compounds for herbicidal properties. The method allows reproducible selection from a large number of substances specifically of those having a potent effect in order then to carry out other, more intensive tests which are familiar to the skilled worker on these substances.

A further aspect of the invention is a method for identifying substances having a herbicidal action, which inhibit the GMP synthetase activity in plants, consisting of

a) preparation of transgenic plants, plant tissues, or plant cells which comprise an additional DNA sequence coding for an enzyme having GMP synthetase activity and are able to overexpress an enzymatically active GMP synthetase;

b) application of a substance to transgenic plants, plant cells, plant tissues or plant parts and to untransformed plants, plant cells, plant tissues or plant parts;

c) determination of the growth or survivability of the transgenic and untransformed plants, plant cells, plant tissues or plant parts after application of the chemical substance; and

d) comparison of the growth or survivability of the transgenic and untransformed plants, plant cells, plant tissues or plant parts after application of the chemical substance;

where suppression of the growth or survivability of the untransformed plants, plant cells, plant tissues or plant parts without, however, greatly suppressing the growth or the survivability of the transgenic plants, plant cells, plant tissues or plant parts demonstrates that the substance from b) shows herbicidal activity and inhibits the GMP synthetase enzymic activity in plants.

A further aspect of the invention is a method for identifying inhibitors of plant GMP synthetases, with potential herbidical action, by cloning the gene of a plant GMP synthetase, bringing about overexpression in a suitable expression cassette - for example in insect cells, opening the cells and employing the cell extract directly or after concentration or isolation of the enzyme GMP synthetases in an assay system for measuring the enzymic activity in the presence of low molecular weight chemical compounds.

A further aspect of the invention comprises compounds having a herbicidal action which can be identified using the assay system described above.

A further aspect of the invention is a method for eliminating unwanted plant growth, which comprises treating the plants to be eliminated with a compound which specifically binds to plant GMP

synthetase and inhibits the function thereof.

Inhibitors of GMP synthetase with a herbicidal action can be used as defoliants, desiccants, haulm destroyers and, in particular, weedkillers. Weeds mean in the widest sense all plants which grow where they are unwanted. Whether the agents found with the aid of the assay system according to the invention act as total or selective herbicides depends inter alia on the application rate.

Inhibitors of GMP synthetase with a herbicidal action can be used, for example, to control the following weeds:

Dicotyledonous weeds of the genera:
Sinapis, Lepidium, Galium, Stellaria, Matricaria, Anthemis, Galinsoga, Chenopodium, Urtica, Senecio, Amaranthus, Portulaca, Xanthium, Convolvulus, Ipomoea, Polygonum, Sesbania, Ambrosia, Cirsium, Carduus, Sonchus, Solanum, Rorippa, Rotala, Lindernia, Lamium, Veronica, Abutilon, Emex, Datura, Viola, Galeopsis, Papaver, Centaurea, Trifolium, Ranunculus, Taraxacum.

Monocotyledonous weeds of the genera:
Echinochloa, Setaria, Panicum, Digitaria, Phleum, Poa, Festuca, Eleusine, Brachiaria, Lolium, Bromus, Avena, Cyperus, Sorghum, Agropyron, Cynodon, Monochoria, Fimbristylis, Sagittaria, Eleocharis, Scirpus, Paspalum, Ischaemum, Sphenoclea, Dactyloctenium, Agrostis, Alopecurus, Apera.

Another aspect of the invention comprises expression cassettes whose sequence codes for a GMP synthetase from tobacco or functional equivalent thereof. The nucleic acid sequence may in this connection be, for example, a DNA or a cDNA sequence.

Another aspect of the invention is an expression cassette comprising a DNA sequence SEQ-ID No. 3 coding for a portion of the plant GMP synthetase from Physcomitrella patens.

The expression cassettes according to the invention additionally comprise regulatory nucleic acid sequences which control the expression of the coding sequence in the host cell. In a preferred embodiment, an expression cassette according to the invention comprises a promoter upstream, i.e. at the 5' end of the coding sequence, and a polyadenylation signal downstream, i.e. at the 3' end, and, where appropriate, further regulatory elements which are operatively linked to the GMP synthetase gene coding sequence lying between them. An operative linkage means tha sequential arrangement of promoter, coding sequence, terminator and, where appropriate, further regulatory elements in

such a way that each of the regulatory elements is able to perform its function as intended on expression of the coding sequence.

An expression cassette according to the invention is produced by fusing a suitable promoter with a suitable GMP synthetase DNA sequence and a polyadenylation signal by conventional recombination and cloning techniques as described, for example, in J. Sambrook et al., Molecular Cloning: A Laboratory Manual, Cold Spring Harbor Laboratory, Cold Spring Harbor, NY (1989) and in T.J. Silhavy, M.L. Berman and L.W. Enquist, Experiments with Gene Fusions, Cold Spring Harbor Laboratory, Cold Spring Harbor, NY (1984) and in Ausubel, F.M. et al., Current Protocols in Molecular Biology, Greene Publishing Assoc. and Wiley-Interscience (1987).

Another aspect of the invention comprises functionally equivalent DNA sequences which code for a GMP synthetase and which have, based on the complete length of the DNA sequence, a sequence homology with the DNA sequence SEQ-ID NO: 1 or SEQ-ID No. 3 of from 40 to 100%.

A preferred aspect of the invention comprises functionally equivalent DNA sequences which code for a GMP synthetase and which have, based on the complete length of the DNA sequence, a sequence homology with the DNA sequence SEQ-ID NO: 1 or SEQ-ID No. 3 of from 60 to 100%.

A particularly preferred aspect of the invention comprises functionally equivalent DNA sequences which code for a GMP synthetase and which have, based on the complete length of the DNA sequence, a sequence homology with the DNA sequence SEQ-ID NO: 1 or SEQ-ID No. 3 of from 80 to 100%.

Functionally equivalent sequences which code for a GMP synthetase are, according to the invention, sequences which, despite a different nucelotide sequence, still have the desired functions. Functional equivalents thus comprise naturally occurring variants of the sequences described herein, and artificial nucleotide sequences, for example obtained by chemical synthesis, which are adapted to the codon usage of a plant.

A functional equivalent also means in particular natural or artificial mutations of an originally isolated sequence which codes for a GMP synthetase and additionally shows the required function. Mutations comprise substitutions, additions, deletions, transpositions or insertions of one or more nucleotide residues.

Thus, for example, the present invention also includes nucleotide sequences obtained by modification of the nucleotide sequence. The aim of such a modification may be, for example, further localization of the coding sequence present therein or, for example, insertion of further restriction enzyme cleavage sites.

Functional equivalents are also variants whose function has been attenuated or enhanced by comparison with initial gene or gene fragment.

The expression cassette according to the invention can also be employed for transforming bacteria, cyanobacteria, yeasts, filamentous fungi and algae with the aim of producing adequate amounts of the enyzme GMP synthetase.

A further aspect of the invention is a protein from tobacco which has the amino acid sequence SEQ-ID NO: 2 or derivatives or portions of this protein with GMP synthetase activity.

Another aspect of the invention comprises plant proteins with GMP synthetase activity having an amino acid sequence homology with the tobacco GMP synthetase of 20 - 100% identity.

Preferred plant proteins with GMP synthetase activity have an amino acid sequence homology with the tobacco GMP synthetase of 50 - 100% identity.

Particularly preferred plant proteins with GMP synthetase activity have an amino acid sequence homology with the tobacco GMP synthetase of 80 - 100% identity.

Another aspect of the invention comprises plant proteins with GMP synthetase activity having an amino acid sequence homology with the Physcomitrella patens GMP synthetase of 20 - 100% identity.

Preferred plant proteins with GMP synthetase activity have an amino acid sequence homology with the Physcomitrella patens GMP synthetase of 50 - 100% identity.

Particularly preferred plant proteins with GMP synthetase activity have an amino acid sequence homology with the Physcomitrella patens GMP synthesase of 80 - 100% identity.

A further object of the invention was overexpression of the GMP synthetase gene in plants to produce plants which are tolerant of inhibitors of GMP synthetase.

Overexpression of the gene sequence SEQ-ID NO: 1 coding for a GMP synthetase in a plant achieves increased resistance to inhibitors of GMP synthetase. The transgenic plants produced in this way are likewise an aspect of the invention.

The efficiency of expression of the transgenically expressed GMP synthetase gene can be measured, for example, in vitro by shoot meristem propagation or by a germination test. In addition, a change in nature and level of the expression of the GMP synthetase gene and the effect thereof on the resistance to inhibitors of GMP synthetase can be tested on test plants in glasshouse experiments.

An additional aspect of the invention comprises transgenic plants transformed with an expression cassette according to the invention comprising DNA sequence SEQ-ID No. 1, which has become tolerant of inhibitors of GMP synthetase due to additional expression of DNA sequence SEQ-ID No. 1, and to transgenic cells, tissues, parts and propagation material of such plants. Particular preference is given in this connection to transgenic crop plants such as, for example, barley, wheat, rye, corn, soybean, rice, cotton, sugar beet, canola, sunflower, flax, hemp, potato, tobacco, tomato, oilseed rape, alfalfa, lettuce and the various tree, nut and vine species, and legumes.

An alteration in the nucleotide content in plants may be beneficial in various cases. For example, nucleotides are added to plant-based babyfood products in order to achieve a nutrient solution corresponding to breast milk. In addition, an optimized nucleotide content would be sensible in the enteral feeding of patients. A reduced purine nucleotide content in plants of foods relevance is relevant to the dietary feeding of patients with gout. Nucleotides also have flavor-forming and flavor-enhancing effects so that an altered nucleotide content has effects on the taste properties of plants.

A further aspect of the invention therefore comprises plants which have a modified content of guanosine nucleotides after expression of the DNA sequence SEQ-ID NO: 1 or SEQ-ID No: 3 in the plant.

A plant with a modified content of guanosine nucleotides is based, for example, by expression of an additional DNA sequence SEQ-ID No. 1 or 3 in the sense or antisense orientation in the plant. A modified content of guanosine nucleotides means that it is possible to produce plants with an increased content of guanosine nucleotides in the case of the sense orientation and

plants with a reduced content of guanosine nucleotides in the case of the sense orientation (cosuppression) or antisense orientation.

Increasing the content of guanosine nucleotides means, for example, within the scope of the present invention the artificially applied capability of increased biosynthesis of guanosine nucleotides owing to functional overexpression of the GMP synthetase gene in the plant compared with the plant which has not been genetically manipulated for the duration of at least one plant generation.

A further aspecet of the invention is the use of plant GMP synthetases to alter the concentrations of methylxanthines in plants.

Particularly preferred sequences are those which ensure targeting in the apoplasts, in plastids, the vacuoles, the mitochondrion, the endoplasmic reticulum (ER) or, through the absence of appropriate operative sequences, ensure retention in the compartment of production, the cytosol (Kermode, Crit. Rev. Plant Sci. 15, 4 (1996), 285-423).

For example, the plant expression cassette can be incorporated into the tobacco transformation vector pBinAR, see Example 6.

Suitable in principle as promoter for the expression cassette according to the invention is every promoter able to control expression of foreign genes in plants. It is particularly preferred to use a plant promoter or a promoter derived from a plant virus. The CaMV 35S promoter from Blumenkohl mosaic virus (Franck et al., Cell 21 (1980), 285-294) is particuliarly preferred. This promoter contains various recognition sequences for transcriptional effectors which, in their totality, lead to permanent and constitutive expression of the introduced gene (Benfey et al., EMBO J. 8 (1989), 2195-2202).

The expression cassette according to the invention may also comprise a chemically inducible promoter through which it is possible to control expression of the exogenous GMP synthetase gene in the plant at a particular point in time. Promoters of this type, such as, for example, PRP1 promoter (Ward et al., Plant. Mol. Biol. (1993) 22, 361-366), a promoter inducible b y salicylic acid (WO 95/19443), a benzenesulfonamide-inducible (EP 388186), a tetracycline-inducible (Gatz et al., Plant J. (1992) 2, 397-404), an abscisic acid-inducible (EP0335528) or an ethanol- or cyclohexanone-inducible (WO 93/21334) promoter are

described in the literature and can, inter alia, be used.

Further particularly preferred promoters are those which ensure expression in tissues or plant parts in which the biosynthesis of purines or their precursors takes place. Particular mention may be made of promoters which ensure leaf-specific expression. Mention should also be made of the promoter of the potato cytosolic FBPase or the potato ST-LSI promoter (Stockhaus et al., EMBO J., (1989) 8, 2445-2451).

It is possible with the aid of a seed-specific promoter to express a foreign protein stably up to a content of 0.67% of the total soluble seed protein in the seed of transgenic tobacco plants (Fiedler and Conrad, Bio/Technology (1995) 10, 1090-1094). The expression cassette according to the invention can therefore comprise, for example, a seed-specific promoter (preferably the phaseolin promoter, the USP or LEB4 promoter), the LEB4 signal peptide, the gene to be expressed and an ER retention signal.

The inserted nucleotide sequence coding for a GMP synthetase can be prepared synthetically or be obtained naturally or comprise a mixture of synthetic and natural DNA components. In general, synthetic nucleotide sequences are produced with codons preferred by plants. These codons preferred by plants can identified by codons which have the highest protein frequency and are expressed in most plant species of interest. To prepare an expression cassette it is possible to manipulate various DNA fragments in order to obtain a nucleotide sequence which expediently reads in the correct direction and is equipped with a correct reading frame. Adaptors or linkers can be attached to the framework to connect the DNA fragments together.

Artificial DNA sequences are also suitable as long as they confer, as described above, the required property of increasing the content of guanosine nucleotides in the plant through overexpression of the GMP synthetase gene in crop plants. Such artificial DNA sequences can be found, for example, by translation back from proteins having GMP synthetase activity and constructed by molecular modeling, or by *in vitro* selection. Particularly suitable coding DNA sequences are those obtained by translation back from a polypeptide sequence in accordance with the codon usage specific for the host plant. The specific codon usage can easily be found by a skilled worker familiar with methods of plant genetics by computer analyses of other known genes of the plant to be transformed.

Further suitable equivalent nucleic acid sequences according to the invention which may be mentioned are sequences coding for fusion proteins where one constituent of the fusion protein is a plant GMP synthetase polypeptide or a functionally equivalent portion thereof. The second part of the fusion protein can be, for example, another polypeptide with enzymatic activity or an antigenic polypeptide sequence with whose aid it is possible to detect GMP synthetase expression (e.g. myc tag or his tag). However, this is preferably a regulatory protein sequence such as, for example, a signal or transit peptide which guides the GMP synthetase protein to the desired site of action.

The promoter regions according to the invention and the terminator regions ought expediently to be provided in the direction of transcription of a linker or polylinker containing one or more restriction sites for insertion of this sequence. As a rule, the linker has 1 to 10, usually 1 to 8, preferably 2 to 6, restriction sites. The size of the linker within the regulatory region is generally less than 100 bp, frequently less than 60 bp, but at least 5 bp. The promoter according to the invention may be both native or homologous and foreign or heterologous to the host plant. The expression cassette according to the invention comprises in the 5'-3' direction of transcription the promoter according to the invention, any suitable sequence and a region for the transcriptional termination. Different termination regions can be exchanged for one another if desired.

It is furthermore possible to employ manipulations which provide suitable restriction cleavage sites or delete the excess DNA or restriction cleavage sites. Where the insertions, deletions or substitutions such as, for example, transitions and transversions are considered, it is possible to use *in vitro* mutagenesis, primer repair, restriction or ligation. In the case of suitable manipulations such as, for example, restriction, chewing-back or filling in of overhangs for blunt ends, it is possible to make complementary ends of the fragments available for the ligation.

Preferred polyadenylation signals are plant polyadenylation signals, preferably those which essentially correspond to T-DNA polyadenylation signals from *Agrobacterium tumefaciens*, in particular of gene 3 of the T-DNA (octopine synthase) of Ti plasmid pTiACH5 (Gielen et al., EMBO J. 3 (1984) 835 ff) or functional equivalents.

To transform a host plant with a DNA coding for a GMP synthetase, an expression cassette according to the invention is incorporated

as insert into a recombinant vector whose vector DNA contains additional functional regulatory signals, for example sequences for replication or integration. Suitable vectors are described inter alia in "Methods in Plant Molecular Biology and Biotechnology" (CRC Press), Chapters 6/7, pages 71-119.

The transfer of foreign genes into the genome of a plant is referred to as transformation. The methods used for this purpose are those described for the transformation and regeneration of plants from plant tissues or plant cells for transient or stable transformation. Suitable methods are protoplast transformation by polyethylene glycol-induced DNA uptake, the biolistic approach with the gene gun, electroporation, incubation of dry embryos in DNA-containing solution, microinjection and gene transfer mediated by agrobacterium. The methods mentioned are described, for example, in B. Jenes et al., Techniques for Gene Transfer, in: Transgenic Plants, Vol. 1, Engineering and Utilization, edited by S.D. Kung and R. Wu, Academic Press (1993) 128-143 and in Potrykus, Annu. Rev. Plant Physiol. Plant Molec. Biol. 42 (1991) 205-225). The construct to be expressed is preferably cloned into a vector which is suitable for transforming *Agrobacterium tumefaciens*, for example pBin19 (Bevan et al., Nucl. Acids Res. 12 (1984) 8711).

Agrobacteria transformed with an expression cassette according to the invention can likewise be used in known manner for transforming plants, especially plants such as cereals, corn, soybean, rice, cotton, sugarbeet, canola, sunflower, flax, hemp, potato, tobacco, tomato, oilseed rape, alfalfa, lettuce and various tree, nut and vine species, and legumes, for example by bathing wounded leaves or pieces of leaf in a solution of agrobacteria and then cultivating in suitable media.

The site of pyrimidine biosythesis is generally the leaf tissue, so that leaf-specific expression of the GMP synthetase gene is sensible. However, it is obvious that pyrimidine biosynthesis need not be confined to leaf tissue but may also take place in all other parts of the plant, for example in fat-containing seeds, tissue-specifically.

In addition, constitutive expression of the exogenous GMP synthetase gene is advantageous. However, on the other hand, inducible expression may also appear desirable.

Using the recombinant and cloning techniques quoted above, the expression cassettes according to the invention can be cloned into suitable vectors which make it possible to replicate them,

for example into E. coli. Suitable cloning vectors are, inter alia, pBR322, pUC series, M13mp series and pACYC184. Binary vectors able to replicate both in E. coli and in agrobacteria are particularly suitable.

A further aspect of the invention relates to the use of an expression cassette according to the invention for transforming plants, plant cells, plant tissues or parts of plants. The aim of the use is preferably to increase the GMP synthetase content in the plant.

This may involve, depending on the chosen promoter, expression specifically in the leaves, in the seeds or other parts of the plant. Such transgenic plants, their propagation material and their plant cells, tissues or parts form a further aspect of the present invention.

The invention is illustrated by the Examples which now follow, but is not confined to these:

Examples

Methods of genetic manipulation on which the examples are based:

General cloning methods

Cloning methods such as, for example, restriction cleavage, agarose gel electrophoresis, purification of DNA fragments, transfer of nucleic acids to nitrocellulose and nylon membranes, linkage of DNA fragments, transformation of Escherichia coli cells, cultivation of bacteria and sequence analysis of recombinant DNA were carried out as described by Sambrook et al. (1989) (Cold Spring Harbor Laboratory Press: ISBN 0-87969-309-6).

Sequence analysis of recombinant DNA

Recombinant DNA molecules were sequenced using a laser fluorescence DNA sequencer supplied by ABI by the method of Sanger (Sanger et al. (1977), Proc. Natl. Acad. Sci. USA74, 5463-5467). Fragments resulting from a polymerase chain reaction were sequenced and checked to avoid polymerase errors in constructs to be expressed.

The chemicals used were purchased, unless otherwise mentioned, in analytical quality from Fluka (Neu-Ulm), Merck (Darmstadt), Roth (Karlsruhe), Serva (Heidelberg) and Sigma (Deisenhofen). Solutions were made up using prepared, pyrogen-free water,

referred to as H_2O in the subsequent text, from a Milli-Q water treatment system (Millipore, Eschborn). Restriction endonucleases, DNA-modifying enzymes and molecular biology kits were purchased from AGS (Heidelberg), Amersham (Braunschweig), Biometra (Göttingen), Roche (Mannheim), Genomed (Bad Oeynhausen), New England Biolabs (Schwalbach/Taunus), Novagen (Madison, Wisconsin, USA), Perkin-Elmer (Weiterstadt), Pharmacia (Freiburg), Qiagen (Hilden) and Stratagene (Heidelberg). They were used in accordance with the manufacturers' instruction unless mentioned otherwise.

The strains of bacteria used hereinafter (E. coli, XL-1 Blue) were purchased from Stratagene. E. coli AT 2465 was purchased from the coli genetic stock center (Yale University, New Haven). The agrobacterial strain used for the plant transformation (Agrobacterium tumefaciens, C58C1 with the plasmid pGV2260 or pGV3850kan) has been described by Deblaere et al. (Nucl. Acids Res. 13 (1985) 4777). An alternative possibility is also to employ the agrobacterial strain LBA4404 (Clontech) or other suitable strains. Vectors which can be used for the cloning are pUC19 (Yanish-Perron, Gene 33(1985), 103-119) pBluescript SK- (Stratagene), pGEM-T (Promega), pZerO (Invitrogen), pBin19 (Bevan et al., Nucl. Acids Res. 12(1984) 8711-8720) and pBinAR (Höfgen and Willmitzer, Plant Science 66 (1990) 221-230).

Example 1

Isolation of a cDNA of the guaA gene, coding for a GMP synthetase from tobacco.

An expressed sequence tag (EST) from Arabidopsis thaliana (EST F14426) which, on a partial reading frame, encodes a polypeptide of 68 amino acids with 60% similarity with a GMP synthetase from *Helicobacter pylori* was subjected to partial 5'-terminal sequencing. The oligonucleotides 5'-aag gat cca agc tct aag acc cta tcc-3' and 5'-tta gat ctt tat tcc cat tcg atg g-3' from the 5'- and 3'-terminal sequences were used for amplification by a polymerase chain reaction (PCR) of a 1000 bp cDNA fragment with EST F14426 as template in a Perkin Elmer DNA thermal cycler. The reaction mixture contained 0.1 ng/µl cDNA from tobacco, 0.5 µM of the appropriate oligonucleotides, 200 µM nucleotides (Pharmacia), 50 mM KCl, 10 mM Tris-HCl (pH 8.3 at 25°C, 1.5 mM $MgCl_2$) and 0.02 U/µl Taq polymerase (Perkin Elmer).

The amplification conditions were set as follows:

Annealing temperature: 52°C, 1 min

Denaturation temperature:	92°C, 1 min
Elongation temperature:	72°C, 1.5 min
Number of cycles:	30

The fragment was employed for screening a cDNA library from callus tissue of *Nicotiana tabacum* (variety Samsun NN) in the vector ZAP Express. For this purpose, 2.5 x 10^5 lambda phages from the cDNA library were plated out on agar plates with E. coli XL1-Blue as bacterial strain. The phage DNA was transferred by standard methods (Sambrook et al. (1989); Cold Spring Harbor Laboratory Press: ISBN 0=87969-309-6) to nitrocellulose filters (Gelman Sciences) and fixed on the filters. The hybridization probe used was the PCR fragment described above which had been radiolabeled using a multiprime DNA labeling system (Amersham Buchler) in the presence of α-^{32}P-dCTP (specific activity 3000 Ci/mmol) in accordance with the manufacturer's information. The hybridization of the membranes took place after prehybridization at 60°C in 3 x SSPE, 0.1% sodium dodecyl sulfate (w/v), 0.02% polyvinylpyrrolidone (w/v), 0.02% Ficoll 400 (w/v) and 50 mg/ml calf thymus DNA for 12-16 hours (Sambrook et al. (1989); Cold Spring Harbor Laboratory Press: ISBN 0-87969-309-6). The filters were then washed in 2 x SSPE, 0.1% sodium dodecyl sulfate (w/v) at 60°C for 60 minutes. Positively hybridizing phages were visualized by autoradiography and purified and isolated by standard techniques.

It was possible to identify and purify 13 hybridizing signals. After restriction analysis, the clones GMP-6 and GMP-7M were selected for double-stranded sequencing. Evaluation of the sequencing data showed that the clone GMP-7M with a length of 1973 bp contained a complete reading frame of 1614 bp which codes for a protein of 538 amino acids with a calculated molecular weight of 60.1 KDa (SEQ-ID No. 1). In front of the presumed start codon there is a stop codon in the same reading frame, which suggests that GMP-7M is a full-length cDNA. GMP-7M thus represents the first full-length plant cDNA of a GMP synthetase. GMP-6 is a partial clone which is 217 nucleotides shorter than GMP-7M on the 5'. GMP-7M shows similarities with GMP synthetases from microorganisms and animals. Apart from the partial amino acid sequence encoded on EST F14426 there are no other sequences from plants with homology with GMP synthetases in the databases. The greatest similarity (62%) is with a GMP synthetase from *Helicobacter pylori*. It is also evident that the similarities between the C termini of the GMP synthetases are greater than those in the region of the N termini. The N terminus of the GMP-7M amino acid sequence corresponds with the N termini of GMP synthetases from other organisms such as *E. coli* and

Synechocystis sp. (Table 1). GMP-7M has no marked signal sequences (found by the program PSORT, Nakai, K., Institute for Molecular and Cellular Biology, Osaka University, Japan), which might indicate a cytosolic localization of the protein.

Table 1

Sequence comparison of GMP synthetases from Nicotiana tabacum (guaA_N.t = GMP-7M), Arabidopsis thaliana (guaA_est_A.t, Genbank No. F14426), E.coli (guaA_e.c, Genbank No. 146276), Synechocystis sp. (guaA_syn, Genbank No. 1001583), Helicobacter pylori (guaA_h.p, Genbank No. 3122166), Homo sapiens (guaA_human, Genbank No. 1708072).

	1				50
guaA_N.t	~~~~~~~~~~	~~~~~~MEPQ	TQAKKSNLVL	ILDYGSQYTH	LITRRIRSLS
guaA_est_A.t	~~~~~~~~~~	~~~~~~~~~~	~~~~~~~~~~	~~~~~~~~~~	~~~~~~~~~~
guaA_e.c	~~~~~~~~~~	~~~~~~~~~m	tenihkhril	ildfgsqytq	lvarrvrelg
guaA_syn	mttqipvppv	vsdqalpdri	sdrlkgqiiv	ildfgsqyse	liarrirete
guaA_h.p	~~~~~~~~~~	~~~~~~~~~~	~~~~~~~mil	vldfgsqytq	liarrlrerg
guaA_human	~malcngdsk	lenaggdlkd	ghhhyegavv	ildagaqygk	vidrrvrelf
	51				100
guaA_N.t	IFSLTINGTS	SLDSIKELDP	RVIILSGGPH	SVHADGAPCF	PPGFIEYVES
guaA_est_A.t	~~~~~~~~~~	~~~~~~~~~~	~~~~~~~~~~	~~~~~~~~~~	~~~~~~~~~~
guaA_e.c	vycelwawdv	teaqirdfnp	sgiilsggpe	stteenspra	pq....yvfe
guaA_syn	vysevlsyrt	taqqlreikp	kgiilsggpn	svydqgapec	dp....eifq
guaA_h.p	iyteivpffe	sieniqkkap	kglilsggpa	svyakdaykp	sg....kifd
guaA_human	vqseifplet	pafaikeqgf	raiiisggpn	svyaedapwf	dpa....ift
	101				150
guaA_N.t	RGIHVLGICY	GLQLIVQKLG	GVVKIGEKHE	YGRMEIEVGK	NVV....GGL
guaA_est_A.t	~~~~~~~~~~	~~~~~~~~~~	~~~~~~~~~~	~~~~~~~~~~	~~~~~~~~~~
guaA_e.c	agvpvfgvcy	gmqtmamqlg	ghveasnere	fgyaqvevvn	dsalvrgied
guaA_syn	lgvpvlgvcy	gmqlmvkqlg	grverakrge	ygkaslhidd	ptdlltnven
guaA_h.p	lnvpilgicy	gmqylvdffg	gvvvganeqe	fgkavleitq	nsvifegv..
guaA_human	igkpvlgicy	gmqmmnkvfg	gtvhkksvre	dgvfnisvdn	tcslfrglqk
	151				200
guaA_N.t	FGNTEIGDKQ	VVWMSHGDEA	VKLPEGFEVV	ARSSQGAVAA	IENRERRFYG
guaA_est_A.t	~~~~~~~~~~	~~~~~~~~~~	~~~~~~~~~~	~~~~~~~~~~	~~~~~~~~~~
guaA_e.c	altadgkpll	dvwmshgdkv	taipsdfitv	astescpfai	maneekrfyg
guaA_syn	dst.......	.mwmshgdsc	vdlptgfeil	ahtdntpcaa	iadhqkalfg
guaA_h.p	......kiks	lvwmshmdkv	ielpkgfttl	akspnsphca	iengk..ifg
guaA_human	........ee	vvllthgdsv	dkvadgfkvv	arsgni.vag	ianeskklyg
	201				250
guaA_N.t	LQYHPEVTHS	TEGMRTLRHF	LFDVCGVTAG	WKMEDVLEEE	IKVIKGMVGP
guaA_est_A.t	~~~~~~~~~~	~~~~~~~~~~	~~~~~~~~~~	~~~~~~~~~~	~~~~~~~~~~
guaA_e.c	vqfhpevtht	rqgmrmlerf	vrdicqceal	wtpakiidda	varireqvg.
guaA_syn	vqfhpevvhs	vggialirnf	vyhichcept	wttaafiees	irevrsqvg.
guaA_h.p	lqfhpevvqs	eeggkilenf	allvcgcekt	wgmqhfaqre	iarlkekia.
guaA_human	aqfhpevglt	engkvilknf	lydiagcsgt	ftvqnrelec	ireikervgt
	251				300
guaA_N.t	EDHVICALSG	GVDSTVAAKL	VHKAIG.DRL	HCVFVDNGLL	RYKERERVME
guaA_est_A.t	~~~~~~~~~~	~~~~~~~~~~	~~~~~~~~~~	~~~~~~~~~~	~~~~~~~~~~
guaA_e.c	ddkvilglsg	gvdssvtaml	lhraig.knl	tcvfvdngll	rlneaeqvld
guaA_syn	drrvllalsg	gvdsstlafl	lhraig.dnl	tcmfidqgfm	rkgeperlve
guaA_h.p	nakvlcavsg	gvdstvvatl	lhraik.dnl	iavfvdhgll	rknekervqa
guaA_human	s.kvlvllsg	gvdstvctal	lnralnqeqv	iavhidngfm	rkresqsvee

```
                301                                                  350
     guaA_N.t   LFEK..... .......... ...RLHLPVT CVDATEEFLS KLKGVTEPEM
 guaA_est_A.t   ---------- ---------- ---------- ---------- ----------
     guaA_e.c   mfgd..... .......... ...hfglniv hvpaedrfls alagendpea
     guaA_syn   lfdh..... .......... ...qfhipvq yvnardrflk qlegvtdpee
     guaA_h.p   mfkd..... .......... ....lkipln tidakevfls klkgvsepel
   guaA_human   alkklgiqvk vinaahsfyn gtttlpisde drtprkrisk tlnmttspee
                351                                                  400
     guaA_N.t   KRKIIGKEFI NIFDLFAHDV EEKVGKKPSY LVQGTLYPDV IESC...PPP
 guaA_est_A.t   ---------- ---------- ---------- ---------- ----------
     guaA_e.c   krkiigrvfv evfd..eeal k...ledvkw laqgtiypdv iesaas....
     guaA_syn   krrlighefi qvfe..eesn r...lgpfdy laqgtlypdv iesadsnvdp
     guaA_h.p   krkiigetfi evfe..keak khhlkgkief laqgtlypdv iesvsv....
   guaA_human   krkiigdtfv ki..anevig emnlkpeevf laqgtlrpdl iesasl....
                401                                                  450
     guaA_N.t   GSGRTHSHTI KSHHNVGGLP KDMKL..KLI EPLKLLFKDE VRELGKILDI
 guaA_est_A.t   ---------- ---------- ---------- ---------- ----------
     guaA_e.c   atgk..ahvi kshhnvgglp kemkm..glv eplkelfkde vrkiglelgl
     guaA_syn   ktgervavki kshhnvgglp knlrf..klv eplrklfkde vrklgrsigl
     guaA_h.p   ...kgpskvi kthhnvgglp ewmdf..kli eplrelfkde vrllgkelgv
   guaA_human   .vasgkaeli kthhndteli rklreegkvi eplkdfhkde vrilgrelgl
                451                                                  500
     guaA_N.t   SEDFLKRHPF PGPGLAVRIP GDVTAGNSLD ILRQVDEIFI QSIRDAKIYD
 guaA_est_A.t   ---------- ---------- ---------- ---------- ----------
     guaA_e.c   pydmlyrhpf pgpglgvrvl gevkk.eycd llrradaifi eelrkadlyd
     guaA_syn   peeivrrhpf pgpglairii gevts.erln ilrdadfivr deiskrgiyh
     guaA_h.p   sqdflmrhpf pgpglavril geise.skik rlqeadfifi eelkkanlyd
   guaA_human   peelvsrhpf pgpglairvi c.aeepyick dfpetnnilk ivadfsasvk
                501                                                  550
     guaA_N.t   EIWQAFAVFL PVKTVGVQGD QRTHSHAVAL RA.VTSQDGM TADWYYFDFK
 guaA_est_A.t   ---------- ------aqgd kgtiphvgcp pcrlqaqvgl tadwfifehk
     guaA_e.c   kvsqaftvfl pvrsvgvmgd grkydwvvsl ra.vetidfm tahwahlpyd
     guaA_syn   dywqafavll pirsvgvmgd krtyahpvvl rf.itsedgm tadwarvpyd
     guaA_h.p   kvwqafcvll nvnsvgvmgd nrtyenaicl ra.vnasdgm tasfsflehs
   guaA_human   kphtllqrvk actteedqek lmqitslhsl naflipiktv gvqgdcrsys
                551                                                  600
     guaA_N.t   FLDDVSRKIC NSVRGVNRVL LDITSKPPST IEWE------ ----------
 guaA_est_A.t   flddvsrkic nsvqgvnrvv lditskppst iewe------ ----------
     guaA_e.c   flgrvsnrii nevngisrvv ydisgkppat iewe------ ----------
     guaA_syn   ileaisnriv nevkgvnrvv yditskppgt iewe------ ----------
     guaA_h.p   flekvsnrit nevsginrvv yditskppgt iewe------ ----------
   guaA_human   yvcgisskde pdweslifla rliprmchnv nrvvyifgpp vkepptdvtp
                601                                                  650
     guaA_N.t   ---------- ---------- ---------- ---------- ----------
 guaA_est_A.t   ---------- ---------- ---------- ---------- ----------
     guaA_e.c   ---------- ---------- ---------- ---------- ----------
     guaA_syn   ---------- ---------- ---------- ---------- ----------
     guaA_h.p   ---------- ---------- ---------- ---------- ----------
   guaA_human   tflttgvlst lrqadfeahn ilresgyagk isqmpviltp lhfdrdplqk
                651                                                  700
     guaA_N.t   ---------- ---------- ---------- ---------- ----------
 guaA_est_A.t   ---------- ---------- ---------- ---------- ----------
     guaA_e.c   ---------- ---------- ---------- ---------- ----------
     guaA_syn   ---------- ---------- ---------- ---------- ----------
     guaA_h.p   ---------- ---------- ---------- ---------- ----------
   guaA_human   qpscqrsvvi rtfitsdfmt gipatpgnei pvevvlkmvt eikkipgisr
                701          716
     guaA_N.t   ---------- ------
 guaA_est_A.t   ---------- ------
     guaA_e.c   ---------- ------
```

```
    guaA_syn  ---------- ------
    guaA_h.p  ---------- ------
  guaA_human  imydltskpp gttewe
```

Example 2

Isolation of a cDNA of the guaA gene, coding for a GMP synthetase from the moss Physcomitrella patens

Double-stranded cDNA was generated from mRNA from protonemata of various ages of Physcomitrella patens and used to produce a cDNA bank in the vector pBluescript SKII (lambda ZAP II RI Library construction kit, Stratagene). Single clones from this bank were partially sequenced. The sequence of the clone 093-d11 showed clear homology with the GMP synthetase from Aquifex aeolicus. The complete sequence of 093-d11 was determined, see SEQ-ID No. 3. 093_d11 has a length of 1232 nucleotides and codes on a continuous reading frame for 382 amino acids. Comparison with GMP-7M reveals that 093_d11 is a partial cDNA. The homology with GMP-7M is 66.7% at the nucleotide level and 74.6% at the amino acid level.

Example 3

Demonstration of the function of GMP-7M by complementation of E. coli

The GMP-7M cDNA was employed as template for a PCR with the oligonucleotides 5'-CCTAGCCATGGAACCTCAAAC-3' and 5'-TATAGGATCCTACTTTGGTCACC-3'. The reaction mixtures contained about 0.1 ng of GMP-7M DNA, 0.5 µM of the appropriate oligonucleotides, 200 µM nucleotides (Pharmacia), 50 mM KCl, 10 mM Tris-HCl (pH 8.3 at 25°C, 1.5 mM $MgCl_2$) and 0.02 U/µl Pfu polymerase (Stratagene).

The amplification conditions were set as follow:

Annealing temperature:	50°C, 30 sec
Denaturation temperature:	92°C, 30 sec
Elongation temperature:	72°C, 3 min
Number of cycles:	25

The resulting fragment of about 1670 bp was ligated via the NcoI and BamHI cleavage sites introduced by the oligonucleotides into the vector pTrc99A (Pharmacia). The resulting construct GMP-7Trc was transformed into the E.coli strain AT2465 (genetic markers: thi-1, guaA21, relA1, λ, spoT1) and plated out on M9 minimal medium (Sambrook et al. (1989) Cold Spring Harbor Laboratory Press: ISBN 0-87969-309-6) with and without 100 µg/ml guanosine.

The minimal media contained 0.4% glucose, 0.2% casamino acids, 100 µg/ml thiamine, 100 µg/ml inosine, 100 µg/ml biotin, 100 µg/ml histidine, 100 µg/ml arginine, 100 µg/ml 2'-deoxyuridine, 100 µM IPTG and 25 µg/ml ampicillin. The cloning vectore pTrc99A was transformed into AT2465 in a parallel experiment. It emerged that only the transformed bacteria which contained a GMP-7M cDNA from tobacco in the expression vector pTrc99A were capable of growth on minimal media without guanosine (see Tab. 2), which points strongly to the GMP-7M cDNA coding for an active GMP synthetase. The enzyme encoded by GMP-7M thus represents the first functional GMP synthetase isolated from plants.

Table 2

Growth of E. coli AT2465 transformed with various plasmids after 2 days at 37°C

	pTrc99A + GMP-7M	pTrc99A
Minimal medium without guanosine	+	-
Minimal medium with guanosine (100 µg/ml)	+	+

Example 4

Overexpression of the GMP synthetase from tobacco in E.coli and production of antibodies

For overexpression in E.coli, BamHI cleavage sites were introduced by PCR with GMP-7M as template and the oligonucleotides GMPA: 5'-GCAATGGATCCTCAAACACAGGCG-3' and GMPB: 5'-AAAAGGATCCTACTTTGGTCACC-3' and made it possible to clone the fragment in the vector pET15b (Novagen). A GMP-7M reading frame with hexahistidine anchor at the N terminus was produced in this way. After the correct orientation had been checked by restriction digestion and polymerase errors had been excluded by sequencing, the resulting construct GMP-7E was transformed into E. coli BL21(DE3) (Stratagene). IPTG-induced one-day cultures were harvested by centrifugation, and the cell pellets were lyzed and treated further in accordance with the manufacturer's information for nickel affinity chromatography ("Qia-Express-Kit", Qiagen). It was possible in this way to purify the GMP synthetase to more than 95% purity. The protein was used for producing antisera in rabbits by conventional

protocols (carried out on contract by Eurogentec, Herstal, Belgium).

Example 5

Expression of the GMP synthetase from tobacco in baculovirus-infected insect cells

In order to obtain sufficient active GMP synthetase for mass testing of chemicals, a 1.65 kb fragment was excized from GMP-7E with BamHI and cloned into the transfer vector pFastBacHTa (GibcoBRL). The resulting construct GMP-7I was used to generate recombinant baculovirus in accordance with the manufacturer's information (GibcoBRL). This virus was used in accordance with the manufacturer's information (GibcoBRL) for infecting Sf21 insect cells in order to produce active GMP synthetase whose activity could be measured after disruption of the cells in 50 mM Tris-HCl, pH 7.6, 10 mM KCl, 1 mM EDTA, 10 mM PMSF and desalting of the extract on a Sephadex G-25 column (Pharmacia, Sweden).

Example 6

Production of plant expression cassettes

The antisense and cosuppression techniques were used with the aim of reducing the GMP synthetase activity in transgenic tobacco plants. For this purpose, plasmid constructs were produced in the vector pBinAR (Höfgen and Willmitzer, Plant Science (1990) 66, 221-230). A fragment of 1599 bp obtained from GMP-7M with *Bam*HI and *Bgl*II was ligated into the *Bam*HI-cut vector pBinAR. The 1599 bp fragment encodes the 5'-terminal part of the GMP synthetase cDNA. Clones obtained after transformation into E.coli XL1-blue were examined for the orientation of the 1599 cassette by cutting with *Hind*III as a check. The plasmids pGMP7AS (antisense construct) and pGMP7EX (sense construct) were identified in this way, see Figure 2.

Example 7

Generation and analysis of transgenic plants

The plasmids pGMP7AS and pGMP7EX - see Figure 2 - were transformed into Agrobacterium tumefaciens C58C1:pGV2260 (Deblaere et al., Nucl. Acids. Res. 13(1984), 4777-4788). A 1:50 dilution of an overnight culture of a positively transformed agrobacterial colony in Murashige-Skoog medium (Physiol. Plant. 15(1962), 473) with 2% sucrose (2MS medium) was used for

transforming tobacco plants (Nicotiana tabacum cv. Samsun NN). Leaf disks from sterile plants (each about 1 cm^2) were incubated in a Petri dish with a 1:50 agrobacteria dilution for 5-10 minutes. This was followed by incubation on 2MS medium with 0.8% Bacto agar in the dark at 25°C for 2 days. Cultivation was continued after 2 days with 16 hours light/8 hours dark, and continued in a weekly rhythm on MS medium with 500 mg/l Claforan (cefotaxime sodium), 50 mg/l kanamycin, 1 mg/l benzylaminopurine (BAP), 0.2 mg/l naphthylacetic acid and 1.6 g/l glucose. Growing shoots were transferred to MS medium with 2% sucrose, 250 mg/l Claforan and 0.8% Bacto agar. Regenerated shoots were obtained on 2MS medium with kanamycin and Claforan and, after rooting, were transferred into soil and, after cultivation for two weeks in a controlled-environment cabinet with a 16 hour light/8 hour dark rhythm at 60% humidity, investigated for foreign gene expression and altered metabolite contents and phenotypical growth traits. Altered nucleotide contents can be determined, for example, by the method of Stitt et al. (FEBS Letters, 145 (1982), 217-222).

The transgenic GMP synthetase antisense plants and their filial generation showed reduced growth, compared with WT plant controls, and bleaching of the sink leaves. These phenotypical changes occurred in an early growth stage (see Fig. 3). In plants with reduced growth it was possible to detect in Northern hybridization a reduced amount of GMP-7M RNA, compared with the wild type. 40 µg portions of complete RNA from sink leaves were employed for this purpose. Complete RNA was isolated from plant tissues as described by Logemann et al. (Anal. Biochem. 163 (1987), 21). For the analysis in each case 40 µg of RNA were fractionated in a formaldehyde-containing 1.5% agarose gel and transferred to nylon membranes (Hybond, Amersham). Specific transcripts were detected as described by Amasino (Anal. Biochem. 152(1986), 304). A specific c-DNA probe of the antisense strand was generated. This was done by cleaving the plasmid GMP-7M with BamHI and BglII and isolating a fragment comprising 1600 bp. The oligonucleotide 5'-GAT ACG TCG TCA AGG AAC TTG-3' was used for the labeling reaction. The probe was hybridized by standard methods, see Hybond information for users, Amersham. Hydridization [sic] signals were visualized by autoradiography using Kodak X-OMAT AR films. A clear correlation between expression of the growth phenotype and a reduction in the amount of GMP-7M RNA was shown (Fig. 3).

It was moreover possible in a Western blot experiment to detect a reduced amount, compared with wild-type plants, of GMP synthetase in the transgenic lines. This was done by preparing total protein extracts from sink leaves, separating in SDS polyacrylamide gel

electrophoresis by standard methods and transferring to nitrocellulose membranes. Detection took place with an IgG-alkaline phosphatase conjugate and the BCIP/NBT system (Sigma).

In addition, it was possible by the in vitro assay described in Example 8 to establish that there was reduced GMP synthetase activity in transgenic lines with reduced growth.

The correlation between the level of expression and the GMP synthetase activity and growth phenotype suggest that GMP synthetase is a suitable target for herbicides.

Example 8

Assay systems for measuring GMP synthetase activity

The systems developed by Spector (Methods in Enzymology LI, 1978, 219-224) for animal enzymes can be used to measure plant GMP synthetase activity. In the first system, the AMP formation is made possible by coupling the reaction with AMP kinase, pyruvate kinase, lactate dehydrogenase and measurement at 340 nm. The second system is based on direct detection of GMP (guanosine monophosphate) by employing the radiolabeled substrate XMP (xanthine monophosphate) and fractionation by thin-layer chromatography.

Alternatively, the GMP synthetase activity can also be measured by a novel system, namely coupled detection of the produced glutamate. This system has the advantage of a smaller number of coupled reaction steps and provides greater signal strengths.

$$\text{XMP + ATP + L-glutamine + } H_2O \xrightarrow{\text{GMP-S}} \text{GMP + AMP + L-glutamate + } PP_i$$

$$\text{L-glutamate + APAD + } H_2O \xrightarrow{\text{GluDH}} \text{oxoglutarate + APADH + } NH_4^+$$

(GMP-S = GMP synthetase, GluDH = glutamate dehydrogenase, APAD = 3-acetylpyridine adenine dinucleotide)

For this, the reaction mixture (see below) was incubated at 37°C for 60 minutes, and the reaction was stopped by incubation at 95°C for 5 minutes. The glutamate formed was detected in the detection mixture (see below) by photometric measurement of the increase in APADH at 363 nm.

Reaction mixture:

100 µL	750 mM	Tris/HCl buffer pH 7.8
100 µL	100 mM	$MgCl_2$
100 µL	80 mM	KCl
100 µL	20 mM	XMP
100 µL	200 mM	L-glutamine
400 µL		H_2O
100 µL		protein extract
1000 µL		

Detection mixture:

375 µL	100 mM	Tris-HCl buffer pH 8.0
75 µL	500 mM	KCl
125 µL		H_2O
75 µL	3 mM	APAD
100 µL		of the reaction mixture
750 µL		

Example 9

Search for inhibitors of GMP synthetase activity

The *in vitro* assay described in Example 8 can be used with high throughput methods to search for inhibitors of GMP synthetase activity. The GMP synthetase activity for this can be prepared from plant tissues. It is possible and preferred for a plant GMP synthetase to be expressed in E.coli, insect cells or another suitable expression system and then be concentrated or isolated. It was possible in this way to identify known inhibitors such as 6-thio-XMP.

10/088027
JC13 Rec'd PCT/PTO 14 MAR 2002

We claim:

1. A DNA sequence comprising the coding region of a plant GMP synthetase, wherein this DNA sequence has the nucleotide sequence SEQ-ID No: 1 or SEQ-ID No: 3.

2. A DNA sequence which hybridizes with the DNA sequence SEQ-ID No: 1 or SEQ-ID No: 3 as claimed in claim 1 or parts thereof or derivatives derived from these sequences by insertion, deletion or substitution, and codes for a protein which has the biological activity of a GMP synthetase, this DNA sequence having a homology of at least 60% with SEQ ID NO: 1.

3. A protein having GMP synthetase activity and comprising an amino acid sequence which represents a portion of at least 100 amino acids of the sequence SEQ-ID No: 2 or 4.

4. A protein as claimed in claim 3, which comprises as amino acid sequence the part-sequence 50 - 300 from SEQ-ID No: 2 or SEQ-ID No: 4.

5. A protein as claimed in claim 4, which comprises as amino acid sequence the sequence depicted in SEQ-ID No: 2 or SEQ-ID No: 4.

6. The use of a DNA sequence as claimed in claim 1 or 2 for introduction into pro- or eukaryotic cells, this sequence optionally being linked to control elements which ensure transcription and translation in the cells, and leading to expression of a translatable mRNA which brings about the synthesis of a plant GMP synthetase.

7. The use of a DNA sequence as claimed in claim 1 or 2 for producing an assay system for identifying inhibitors of plant GMP synthetase with a herbicidal action.

8. A method for finding substances which inhibit the activity of plant GMP synthetase, which comprises in a first step using a DNA sequence as claimed in claim 1 or 2 preparing GMP synthetase, and in a second step measuring the activity of the plant GMP synthetase in the presence of a test substance.

9. A method as claimed in claim 8, wherein the measurement of the plant GMP synthetase is carried out in a high throughput screening (HTS).

10. A method for identifying substances with a herbicidal action, which inhibit the GMP synthetase activity in plants, consisting of

a) preparation of transgenic plants, plant tissues, or plant cells which comprise an additional DNA sequence coding for an enzyme having GMP synthetase activity and are able to overexpress an enzymatically active GMP synthetase;

b) application of a substance to transgenic plants, plant cells, plant tissues or plant parts and to untransformed plants, plant cells, plant tissues or plant parts;

c) determination of the growth or survivability of the transgenic and untransformed plants, plant cells, plant tissues or plant parts after application of the chemical substance; and

d) comparison of the growth or survivability of the transgenic and untransformed plants, plant cells, plant tissues or plant parts after application of the chemical substance;

where suppression of the growth or survivability of the untransformed plants, plant cells, plant tissues or plant parts without, however, greatly suppressing the growth or the survivability of the transgenic plants, plant cells, plant tissues or plant parts demonstrates that the substance from b) shows herbicidal activity and inhibits the enzymic activity in plants.

11. An assay system based on the expression of a DNA sequence SEQ-ID No. 1 or SEQ-ID No. 3 as claimed in claim 1 or 2 for identifying inhibitors of plant GMP synthetase with a herbicidal action.

12. An assay system as claimed in claim 11 for identifying inhibitors of plant GMP synthetase, wherein the enzyme is incubated with a test substrate to be investigated and, after a suitable reaction time, the enzymatic activity of the enzyme is measured by comparison with the activity of the

uninhibited enzyme.

13. An inhibitor of plant GMP synthetase.

14. An inhibitor of plant GMP synthetase identified using an assay system as claimed in claim 11 or 12.

15. An inhibitor as claimed in either of claims 13 or 14 for use as herbicide.

16. A method for eliminating unwanted plant growth, which comprises treating the plants to be eliminated with a compound which specifically binds to GMP synthetase encoded by a DNA sequence as claimed in claim 1 or 2, and inhibits the function thereof.

GMP synthetase from plants

Abstract

The present invention relates to a DNA coding for a polypeptide having GMP synthetase (EC 6.3.5.2) activity. The invention additionally relates to the use of this nucleic acid for producting an assay system.

FIG. 1

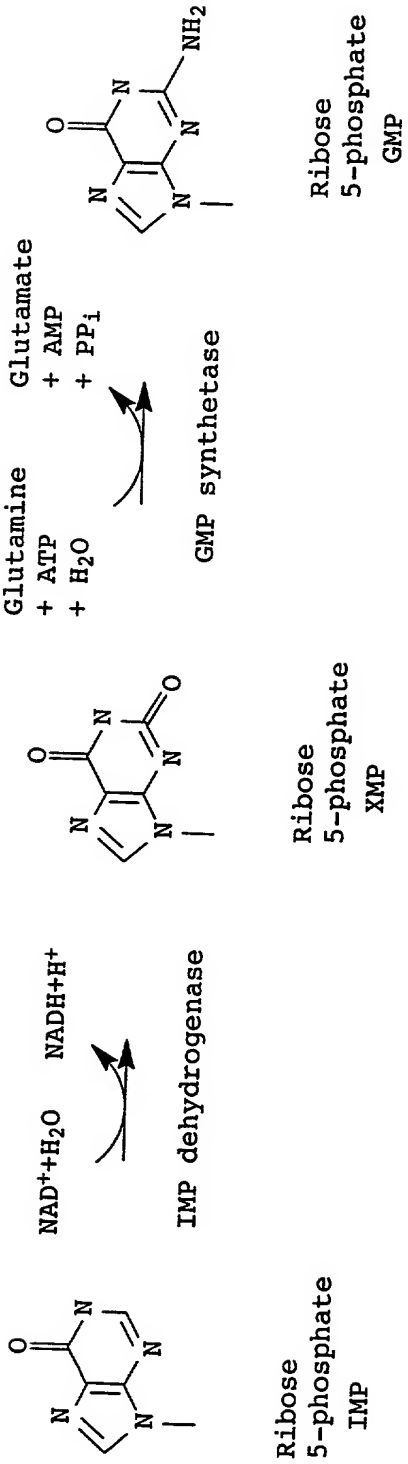
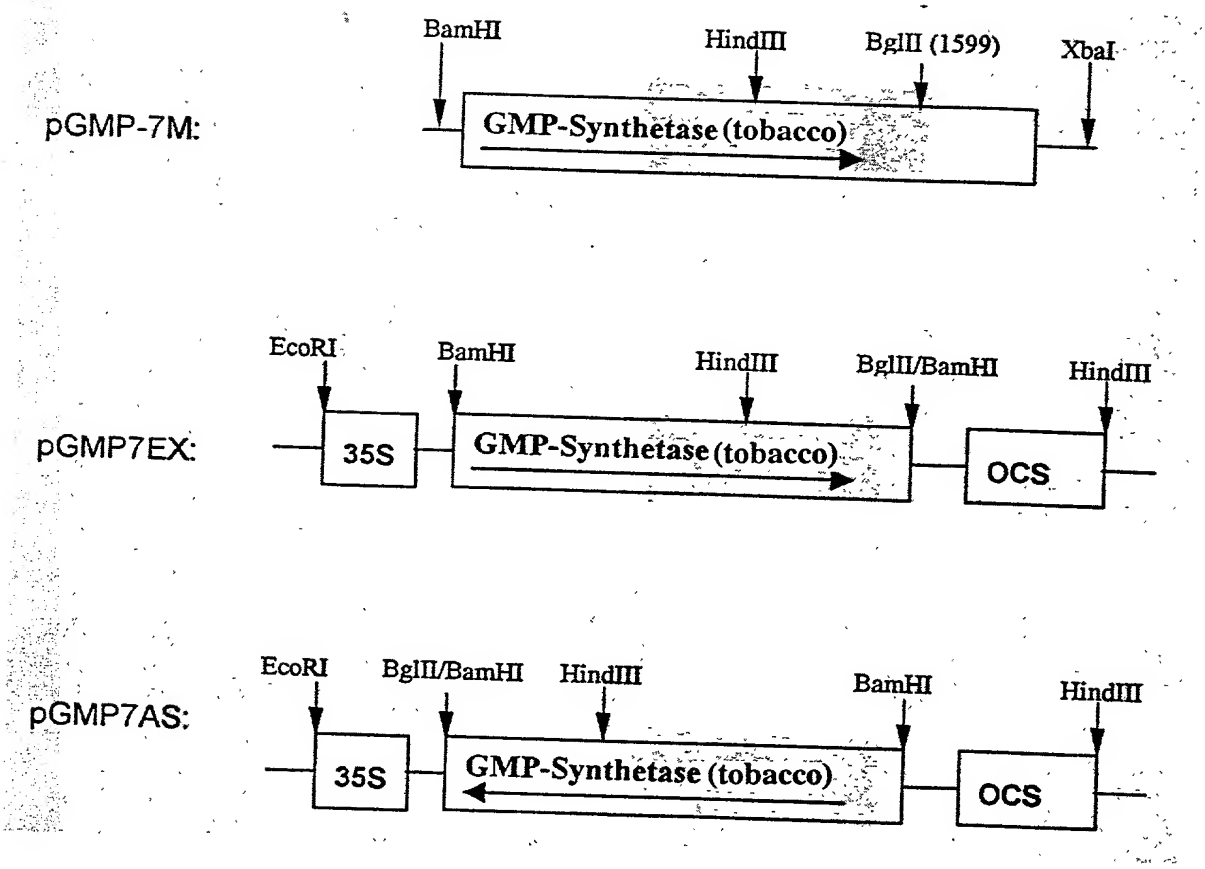


FIG. 2



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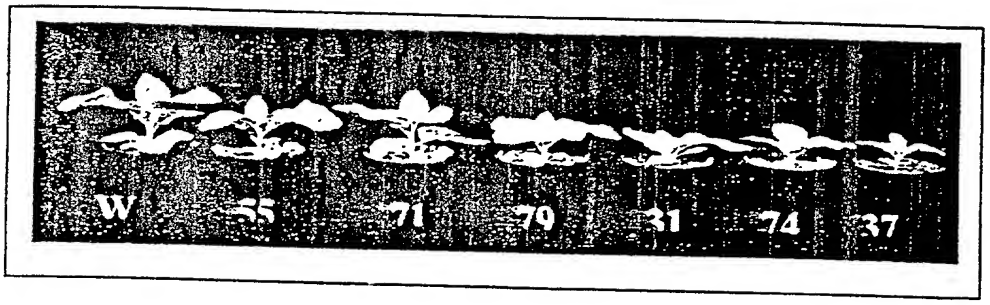
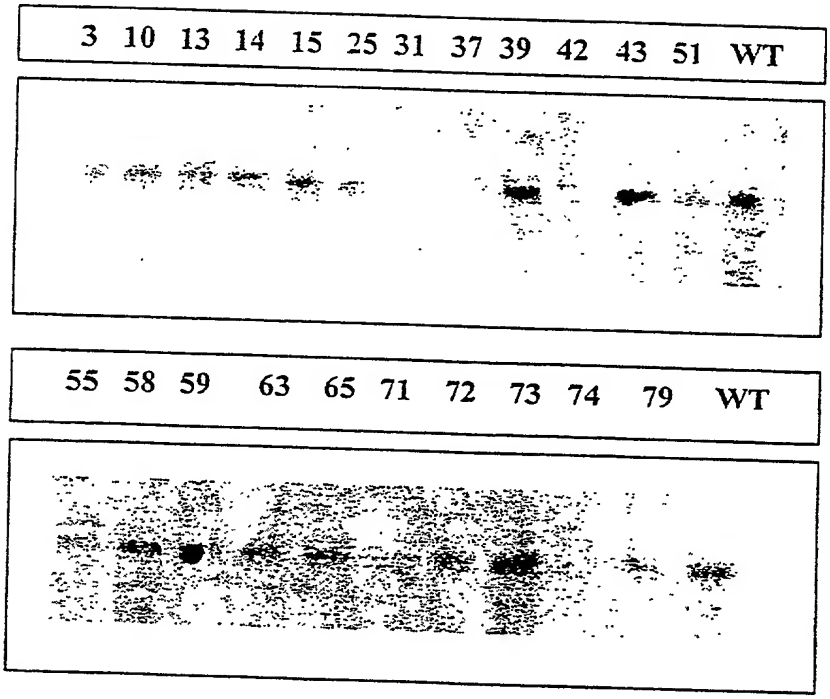


Fig. 4:



Declaration, Power of Attorney and Petition

0050/050777

Customer No.

We (I), the undersigned inventor(s), hereby declare(s) that:

My residence, post office address and citizenship are as stated below next to my name,

We (I) believe that we are (I am) the original, first, and joint (sole) inventor(s) of the subject matter which is claimed and for which a patent is sought on the invention entitled

GMP SYNTHETASE FROM PLANTS

the specification of which

[ ] is attached hereto.

[ ] was filed on _____ as

Application Serial No. _____

and amended on _____ .

[x] was filed as PCT international application

Number *PCT/EP/00/09245*

on *21 September 2000*,

and was amended under PCT Article 19

on _____ (if applicable).

We (I) hereby state that we (I) have reviewed and understand the contents of the above-identified specification, including the claims, as amended by any amendment referred to above.

We (I) acknowledge the duty to disclose information known to be material to the patentability of this application as defined in Section 1.56 of Title 37 Code of Federal Regulations.

We (I) hereby claim foreign priority benefits under 35 U.S.C. § 119(a)-(d) or § 365(b) of any foreign application(s) for patent or inventor's certificate, or § 365(a) of any PCT International application which designated at least one country other than the United States, listed below and have also identified below, by checking the box, any foreign application for patent or inventor's certificate, or PCT International application having a filing date before that of the application on which priority is claimed. Prior Foreign Application(s)

Application No.	**Country**	**Day/Month/Year**	**Priority Claimed**	
19947490.7	*Germany*	*01 October 1999*	[x] Yes	[ ] No

We (I) hereby claim the benefit under Title 35, United States Codes, § 119(e) of any United States provisional application(s) listed below.

(Application Number)	(Filing Date)
(Application Number)	(Filing Date)

We (I) hereby claim the benefit under 35 U.S.C. § 120 of any United States application(s), or § 365(c) of any PCT International application designating the United States, listed below and, insofar as the subject matter of each of the claims of this application is not disclosed in the prior United States or PCT International application in the manner provided by the first paragraph of 35 U.S.C. § 112, I acknowledge the duty to disclose information which is material to patentability as defined in 37 CFR § 1.56 which became available between the filing date of the prior application and the national or PCT International filing date of this application.

Application Serial No.	**Filing Date**	**Status (pending, patented, abandoned)**

And we (I) hereby appoint **Nixon & Vanderhye P.C.**, Attorneys at Law, 1100 North Glebe Road, Arlington, Virginia 22201-4714, our attorneys, with full power of substitution and revocation, to prosecute this application, to make alterations and amendments therein, to sign the drawings, to receive the patent, and to transact all business in the Patent Office connected therewith.

We (I) declare that all statements made herein of our (my) own knowledge are true and that all statements made on information and belief are believed to be true; and further that these statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under Section 1001 of Title 18 of the United States Code and that such willful false statements may jeopardize the validity of the application or any patent issuing thereon.

Jens Lerchl
NAME OF INVENTOR

1st

Signature of Inventor

Date October 5, 2000

Im Steg 36
68526 Ladenburg DEX
Germany
Citizen of: Germany
Post Office Address: same as residence

Thomas Ehrhardt
NAME OF INVENTOR

2nd

Signature of Inventor

Date October 5, 2000

Maulbronner Hof 49
67346 Speyer DEX
Germany
Citizen of: Germany
Post Office Address: same as residence

Uwe Sonnewald
NAME OF INVENTOR

3rd

Signature of Inventor

Date October 5, 2000

Am Hange 6
06484 Quedlinburg DEX
Germany
Citizen of: Germany
Post Office Address: same as residence

Ralf Boldt
NAME OF INVENTOR

4th

Signature of Inventor

Date October 5, 2000

Stieg 19
06484 Quedlinburg DEX
Germany
Citizen of: Germany
Post Office Address: same as residence

SEQUENCE LISTING

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gag att aaa aag gtc act gcg acc gtc ggc cca gat gat cat gtt att   239
Glu Ile Lys Lys Val Thr Ala Thr Val Gly Pro Asp Asp His Val Ile
     65                  70                  75

tgt gca ctc tcc ggg ggc gtg gac tca aca gta gca gct act ctg gtg   287
Cys Ala Leu Ser Gly Gly Val Asp Ser Thr Val Ala Ala Thr Leu Val
 80                  85                  90                  95

cac cgt gct att gga gat cgc ctt cat tgt gtg ttt gta gat aat ggc   335
His Arg Ala Ile Gly Asp Arg Leu His Cys Val Phe Val Asp Asn Gly
                100                 105                 110

ctt tgc aga tac aag gaa aga gaa aga gtg atg gcc aca ttt gtg aaa   383
Leu Cys Arg Tyr Lys Glu Arg Glu Arg Val Met Ala Thr Phe Val Lys
            115                 120                 125

gac ctt cat ctg cca gtc act tgt gtg gat gcc act gag cag ttt ctc   431
Asp Leu His Leu Pro Val Thr Cys Val Asp Ala Thr Glu Gln Phe Leu
        130                 135                 140

agc aaa ttg aag ggc gtg gta gat cca gag aga aag agg aag atc atc   479
Ser Lys Leu Lys Gly Val Val Asp Pro Glu Arg Lys Arg Lys Ile Ile
    145                 150                 155

gga gca gag ttt att gca gtc ttt gat gaa ttt tcg cac aga ttg gag   527
Gly Ala Glu Phe Ile Ala Val Phe Asp Glu Phe Ser His Arg Leu Glu
160                 165                 170                 175
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aga gag att gga aag atg cct gct ttc ctt gtg cag gga aca ctt tat   575
Arg Glu Ile Gly Lys Met Pro Ala Phe Leu Val Gln Gly Thr Leu Tyr
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cca gat gtc att gag tcg tgt cct cct cca ggg agc ggg aag tcg cat   623
Pro Asp Val Ile Glu Ser Cys Pro Pro Pro Gly Ser Gly Lys Ser His
            195                 200                 205

tcc cac aca atc aaa agt cat cac aac gtc ggt ggc ttg ccc gag aac   671
Ser His Thr Ile Lys Ser His His Asn Val Gly Gly Leu Pro Glu Asn
        210                 215                 220

atg aaa ttg aag ttg gtt gag cct ctc aag tgg ctc ttc aaa gac gag   719
Met Lys Leu Lys Leu Val Glu Pro Leu Lys Trp Leu Phe Lys Asp Glu
    225                 230                 235

gta cgc gaa atg ggt gca ttg ttg gat gta cct gtt tcc ttt ttg aag   767
Val Arg Glu Met Gly Ala Leu Leu Asp Val Pro Val Ser Phe Leu Lys
240                 245                 250                 255

cgc cat cct ttc cct gga cct gga ttg gcc gtg cga att ctt ggg gat   815
Arg His Pro Phe Pro Gly Pro Gly Leu Ala Val Arg Ile Leu Gly Asp
                260                 265                 270

gta act cag gac ggc gca ctc gac act atc cgc ttg gtt gat gag atc   863
Val Thr Gln Asp Gly Ala Leu Asp Thr Ile Arg Leu Val Asp Glu Ile
            275                 280                 285

ttt gtg aac agc att cga gag gca ggt ctt tac gat aag atc tgg cag   911
Phe Val Asn Ser Ile Arg Glu Ala Gly Leu Tyr Asp Lys Ile Trp Gln
        290                 295                 300

gca ttt gct gtt tat ctg cca gta aag act gtt ggc gtt caa ggc gac   959
Ala Phe Ala Val Tyr Leu Pro Val Lys Thr Val Gly Val Gln Gly Asp
    305                 310                 315

aaa cgg aca cat tca cac gct gtt gct cta cgt gca att aca agt gaa   1007
Lys Arg Thr His Ser His Ala Val Ala Leu Arg Ala Ile Thr Ser Glu
320                 325                 330                 335

gac gga atg act gct gac tgg ttt cat ttt gat gga aag ttt ctt gcc   1055
Asp Gly Met Thr Ala Asp Trp Phe His Phe Asp Gly Lys Phe Leu Ala
                340                 345                 350

gag gta tca tct aaa atc tgc aac agc gta agg ggt atc aat agg gtg   1103
Glu Val Ser Ser Lys Ile Cys Asn Ser Val Arg Gly Ile Asn Arg Val
            355                 360                 365
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gta tac gac att acg tct aaa cct cca tca act gtt gag tgg gaa 1148
Val Tyr Asp Ile Thr Ser Lys Pro Pro Ser Thr Val Glu Trp Glu
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acaaactatt ttatacttca aaaa 1232

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Asn Val Asp Ser Arg Ile Tyr Ala Leu Gln Tyr His Pro Glu Val Thr
20 25 30

His Ser Glu Lys Gly Thr Glu Thr Leu Arg His Phe Phe Leu Asn Val
35 40 45

Cys Gly Met Lys Ala Asp Trp Gln Met Gln Asn Val Leu Glu Glu Glu
50 55 60

Ile Lys Lys Val Thr Ala Thr Val Gly Pro Asp Asp His Val Ile Cys
65 70 75 80

Ala Leu Ser Gly Gly Val Asp Ser Thr Val Ala Ala Thr Leu Val His
85 90 95

Arg Ala Ile Gly Asp Arg Leu His Cys Val Phe Val Asp Asn Gly Leu
100 105 110

Cys Arg Tyr Lys Glu Arg Glu Arg Val Met Ala Thr Phe Val Lys Asp
115 120 125

Leu His Leu Pro Val Thr Cys Val Asp Ala Thr Glu Gln Phe Leu Ser
130 135 140

Lys Leu Lys Gly Val Val Asp Pro Glu Arg Lys Arg Lys Ile Ile Gly
145 150 155 160

Ala Glu Phe Ile Ala Val Phe Asp Glu Phe Ser His Arg Leu Glu Arg
165 170 175

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Glu Ile Gly Lys Met Pro Ala Phe Leu Val Gln Gly Thr Leu Tyr Pro
            180                 185                 190

Asp Val Ile Glu Ser Cys Pro Pro Pro Gly Ser Gly Lys Ser His Ser
        195                 200                 205

His Thr Ile Lys Ser His His Asn Val Gly Gly Leu Pro Glu Asn Met
    210                 215                 220

Lys Leu Lys Leu Val Glu Pro Leu Lys Trp Leu Phe Lys Asp Glu Val
225                 230                 235                 240

Arg Glu Met Gly Ala Leu Leu Asp Val Pro Val Ser Phe Leu Lys Arg
                245                 250                 255

His Pro Phe Pro Gly Pro Gly Leu Ala Val Arg Ile Leu Gly Asp Val
            260                 265                 270

Thr Gln Asp Gly Ala Leu Asp Thr Ile Arg Leu Val Asp Glu Ile Phe
        275                 280                 285

Val Asn Ser Ile Arg Glu Ala Gly Leu Tyr Asp Lys Ile Trp Gln Ala
    290                 295                 300

Phe Ala Val Tyr Leu Pro Val Lys Thr Val Gly Val Gln Gly Asp Lys
305                 310                 315                 320

Arg Thr His Ser His Ala Val Ala Leu Arg Ala Ile Thr Ser Glu Asp
                325                 330                 335

Gly Met Thr Ala Asp Trp Phe His Phe Asp Gly Lys Phe Leu Ala Glu
            340                 345                 350

Val Ser Ser Lys Ile Cys Asn Ser Val Arg Gly Ile Asn Arg Val Val
        355                 360                 365

Tyr Asp Ile Thr Ser Lys Pro Pro Ser Thr Val Glu Trp Glu
    370                 375                 380
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